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Metformin affects the circadian clock and metabolic rhythms in a tissue-specific manner

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ABSTRACT

Metformin is a commonly-used treatment for type 2 diabetes, whose mechanism of action has been linked, in part, to activation of AMP-activated protein kinase (AMPK). However, little is known regarding its effect on circadian rhythms. Our aim was to evaluate the effect of metformin administration on metabolism, locomotor activity and circadian rhythms. We tested the effect of metformin treatment in the liver and muscle of young lean, healthy mice, as obesity and diabetes disrupt circadian rhythms. Metformin led to increased leptin and decreased glucagon levels. The effect of metformin on liver and muscle metabolism was similar leading to AMPK activation either by liver kinase B1 (LKB1) and/or other kinases in the muscle. AMPK activation resulted in the inhibition of acetyl CoA carboxylase (ACC), the rate limiting enzyme in fatty acid synthesis. Metformin also led to the activation of liver casein kinase I α (CKIα) and muscle CKIε, known modulators of the positive loop of the circadian clock. This effect was mainly of phase advances in the liver and phase delays in the muscle in clock and metabolic genes and/or protein expression. In conclusion, our results demonstrate the differential effects of metformin in the liver and muscle and the critical role the circadian clock has in orchestrating metabolic processes.

1. Introduction

The mammalian master circadian clock is located in the hypothalamic suprachiasmatic nuclei (SCN), but there are similar clocks in peripheral tissues [1,2]. The core clock mechanism is composed of the transcription factor CLOCK which interacts with BMAL1 to drive the transcription of a large number of genes. These genes encode the PERIOD (PER1, PER2 and PER3) and CRYPTOCHROME (CRY1 and CRY2) proteins that inhibit CLOCK:BMAL1-mediated transcription, thus serving as the negative feedback loop [2]. Other transcriptional loops involve the control of PER and BMAL1 protein stability by casein kinase 1ε (CKIε) phosphorylation [3]. *Bmal1* expression is also regulated negatively by reverse orientation c-erbA-1 α (REV-ERBα) and positively by receptor-related orphan receptor α (RORα) via the RORE (ROR response element) [4,5].

Metabolism is an important feedback to the circadian clock in the peripheral tissues, synchronizing it to environmental cues, such as food availability [6]. In addition, the core clock mechanism is tightly linked to metabolic pathways: 1) REV-ERBα, RORα, and PPARα (peroxisome proliferator-activated receptor α), regulators of lipogenesis and lipid metabolism, regulate *Bmal1* transcription [4,5,7]. 2) In turn, CLOCK:BMAL1 heterodimer regulates the expression of *Rev-erbα*, *Rorα*, and *Pparα* [4,5,7–9]. 3) PPARγ co-activator 1α (PGC-1α), which regulates energy metabolism, stimulates the expression of the clock genes *Bmal1* and *Rev-erbα*; mice lacking PGC-1α show abnormal diurnal rhythms of activity, body temperature, and metabolic rate [10]. 4) Activation of adenosine monophosphate-activated protein kinase (AMPK), a sensor of low energy and nutrient state in the cell, leads to altered circadian rhythms by destabilizing the negative limb of the circadian clock, PERs and CRYs [11,12].

AMPK is activated when cellular ATP levels are depleted and switches the cell from anabolic to catabolic pathways [13]. Hypothalamic AMPK regulates energy intake by mediating the opposing effects of orexigenic and anorexigenic signals [14]. In the peripheral tissues, AMPK is phosphorylated and activated under elevated AMP levels by liver kinase B1 (LKB1) [15] following hormonal signals, such as adiponectin [16]. Consequently, activated AMPK phosphorylates and, thus, inactivates acetyl-CoA carboxylase (ACC), the key enzyme in fatty acid synthesis. Reduced fatty acid synthesis as well as activation of PPARα result in mitochondrial fatty acid oxidation [16].

Metformin is a commonly-used treatment for type 2 diabetes (T2DM), whose mechanism of action has been linked, in part, to the activation of AMPK [17,18]. However, little is known about its effect on circadian rhythms. As metformin affects metabolism and metabolism is interlinked to the circadian clock, our aim in this study was to evaluate the effect of metformin administration on circadian rhythms.

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As obesity and diabetes affect circadian rhythms [19,20], we assessed the effect of metformin on the metabolic state, locomotor activity and circadian rhythms in the liver and muscle of young lean mice.

2. Methods

2.1. Animals and experimental design

8-week-old C57BL/6 male mice (Harlan Laboratories, Jerusalem, Israel) were housed in a temperature- and humidity-controlled facility (23–24 °C, 60% humidity). Mice were entrained to 12 h light and 12 h darkness (LD) for 2 weeks and then were randomly assigned to either the control (n = 24) or metformin treatment (164 mg/kg/day; n = 24) group for 6 weeks. Metformin was provided in the drinking water designed to achieve the maximum recommended dose of metformin for humans per basal water intake. Body weight was recorded weekly and on the second and seventh week fasting blood glucose levels were determined with a digital "Optium Xceed" glucometer (Abbott Laboratories, Alameda, CA, USA). After 6 weeks of treatment, mice were placed on a 12-h fast and subsequently every 3 h around the circadian cycle animals were tested for blood glucose. CT0/24 time-points are double-plotted in the figures. On the day of sacrifice, mice were anesthetized by intraperitoneal injection of ketamine/xylazine (100/7.5 mg/kg) under dim red light in total darkness (DD). Liver and muscle tissues were removed and immediately frozen in liquid nitrogen and stored at −80 °C for further analysis. Serum was collected and stored at −20 °C for further analyses. Animals were humanely killed according to the strict guidelines of the Hebrew University. The Principles of Laboratory Animal Care (NIH publication no. 85–23, revised 1985) were followed.

2.2. Enzyme-linked immunosorbent assay (ELISA)

Serum hormone levels were determined for ghrelin (Linco Research Inc., St Charles, MO, USA), insulin (Mercodia, Uppsala, Sweden), glucagon (Phoenix Pharmaceuticals Inc., Burlingame, CA, USA) and leptin (R&D Systems Inc., Minneapolis, MN, USA) using ELISA kits. All assays were performed according to the manufacturer's instructions.

2.3. Enzymatic colorimetric test

Serum triglycerides and cholesterol levels were determined by Cobas® kits (Roche Diagnostics, Burgess Hill, UK) and analyzed in a Roche/Hitachi analyzer (Roche Diagnostics). Assays were performed according to the manufacturer's instructions.

Table 1
Biochemical measurements taken on the last day of the experiment.

	Metformin-treated group (n = 24)	Control group (n = 24)	p Value
Body weight	25.2 ± 0.46	24.8 ± 0.51	0.54
Food intake (/kg/day)	0.223 ± 0.003	0.221 ± 0.002	0.61
Water intake (/kg/day)	0.105 ± 0.008	0.082 ± 0.002	0.04
Fasting glucose	69 ± 4.3	61.75 ± 2.9	0.17
Cholesterol	124.9 ± 5.1	122.5 ± 5.5	0.7
HDL	108.6 ± 4	106.3 ± 4.7	0.7
TG	123.3 ± 9.6	131.7 ± 13.3	0.6
Insulin mIU	11.54 ± 0.87	13.15 ± 1.51	0.35
Glucagon (ng/ml)	1.13 ± 0.08	0.53 ± 0.03	<0.0001
HOMA-IR	1.97 ± 0.17	2.18 ± 0.37	0.59
Ghrelin (ng/ml)	2.88 ± 0.26	2.76 ± 0.76	0.76
Leptin (ng/ml)	4.73 ± 0.55	6.77 ± 0.47	0.006
Adiponectin (ng/ml)	304.2 ± 9.5	349.5 ± 15.5	0.018

Values are means ± standard error.
HDL, high density lipoprotein; TG, triglyceride; HOMA-IR, homeostasis model assessment insulin resistance.

2.4. Homeostasis model assessment of insulin resistance (HOMA-IR)

The insulin-resistance index using fasting serum insulin and plasma glucose levels was determined by the homeostasis model assessment (HOMA) parameter [21].

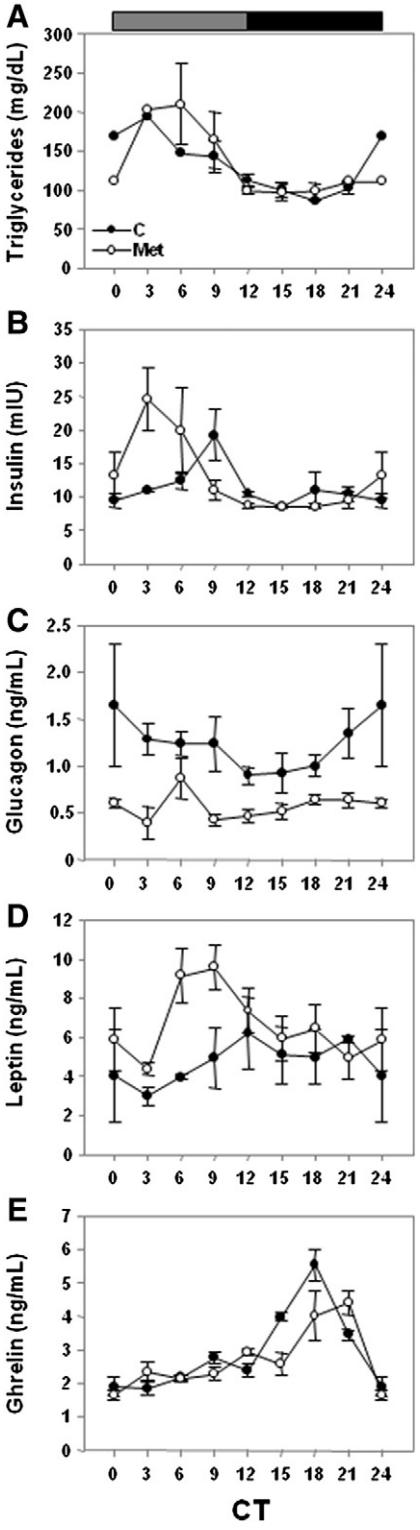


Fig. 1. Effect of metformin on circadian triglycerides and hormone expression. Levels of triglyceride (A), insulin (B), glucagon (C), leptin (D) and ghrelin (E) were determined every 3 h around the circadian cycle in control (C, black circles) or metformin-treated (Met, white circles) mice. The white and gray bars designate the subjective day and night, respectively. Values are mean ± SE, n = 3 per time point in each group. CT, circadian time.

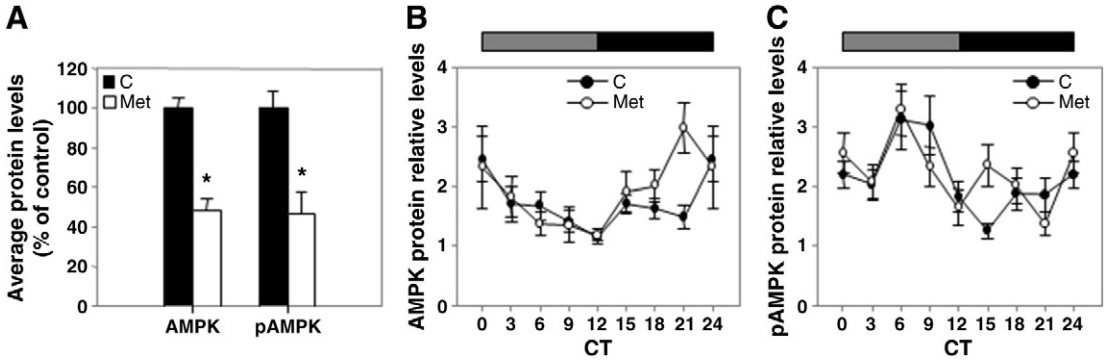


Fig. 2. Effect of metformin on circadian rhythms and average protein levels of AMPK and pAMPK in the hypothalamus. A. Average daily levels of AMPK and pAMPK proteins. B. Circadian AMPK protein expression. C. Circadian pAMPK protein expression. AMPK and pAMPK proteins were analyzed by Western blotting and quantified using actin as loading control. For total daily levels, all time-points were averaged. Hypothalamus was collected in total darkness every 3 h around the circadian cycle from control (C, black circles and columns) or metformin-treated (Met, white circles and columns) mice. The white and gray bars designate the subjective day and night, respectively. Values are mean ± SE, n = 3 per time-point in each group. Asterisk denotes significant difference ($p<0.05$). CT, circadian time.

2.5. RNA extraction and quantitative real-time PCR

RNA extraction and qPCR were performed as was described [22]. Primers (Table S1) were designed with Primer express v.2 (Applied Biosystems, Foster City, CA) and validated by a standard curve and dissociation curve of the product. The fold change in target gene expression was calculated by the $2^{-\Delta\Delta Ct}$ relative quantification method (Applied Biosystems).

2.6. Western blot analysis

Liver tissue samples were homogenized, run on an SDS-polyacrylamide gel and transferred onto nitrocellulose membranes, as was described [22]. Blots were incubated with AMPK/pAMPK, ACC/pACC, LKB1/pLKB1, CK1α antibodies (Cell Signaling Technology, Beverly, MA, USA) and CKIε (Abcam, Cambridge, UK) and after several washes, with horseradish peroxidase-conjugated secondary antibody (Pierce, Rockford, IL, USA). The immune reaction was detected by enhanced chemiluminescence (ECL). Finally, bands were quantified by scanning and densitometry and expressed as arbitrary units.

2.7. Statistical analyses

All results are expressed as means ± SE. A two-way analysis of variance (ANOVA) (time of day × treatment) was performed and a least-significant difference *t*-test was used for comparison between the control and metformin-treated groups. One-way ANOVA was used to analyze circadian pattern with several time-points. Statistical analysis was performed with JMP software (version 8.1, SAS Institute Inc. Cary, NC, USA). The acrophase and p-value of gene oscillation were calculated using the free Cosinor analysis software (Version 2.3) available at http://www.circadian.org/softwar.html. The significance level for all analyses was set at $p<0.05$.

3. Results

Mice were fed regular chow and metformin was added to the drinking water for 6 weeks. As obesity affects circadian rhythms [19,20,23], we used lean healthy mice to measure the effect of metformin without obesity being a confounder. When evaluating metformin effects, we calculated 8 time-points encompassing the entire circadian cycle to achieve an accurate assessment of daily mean levels, as a single point might represent the peak or trough of the circadian expression.

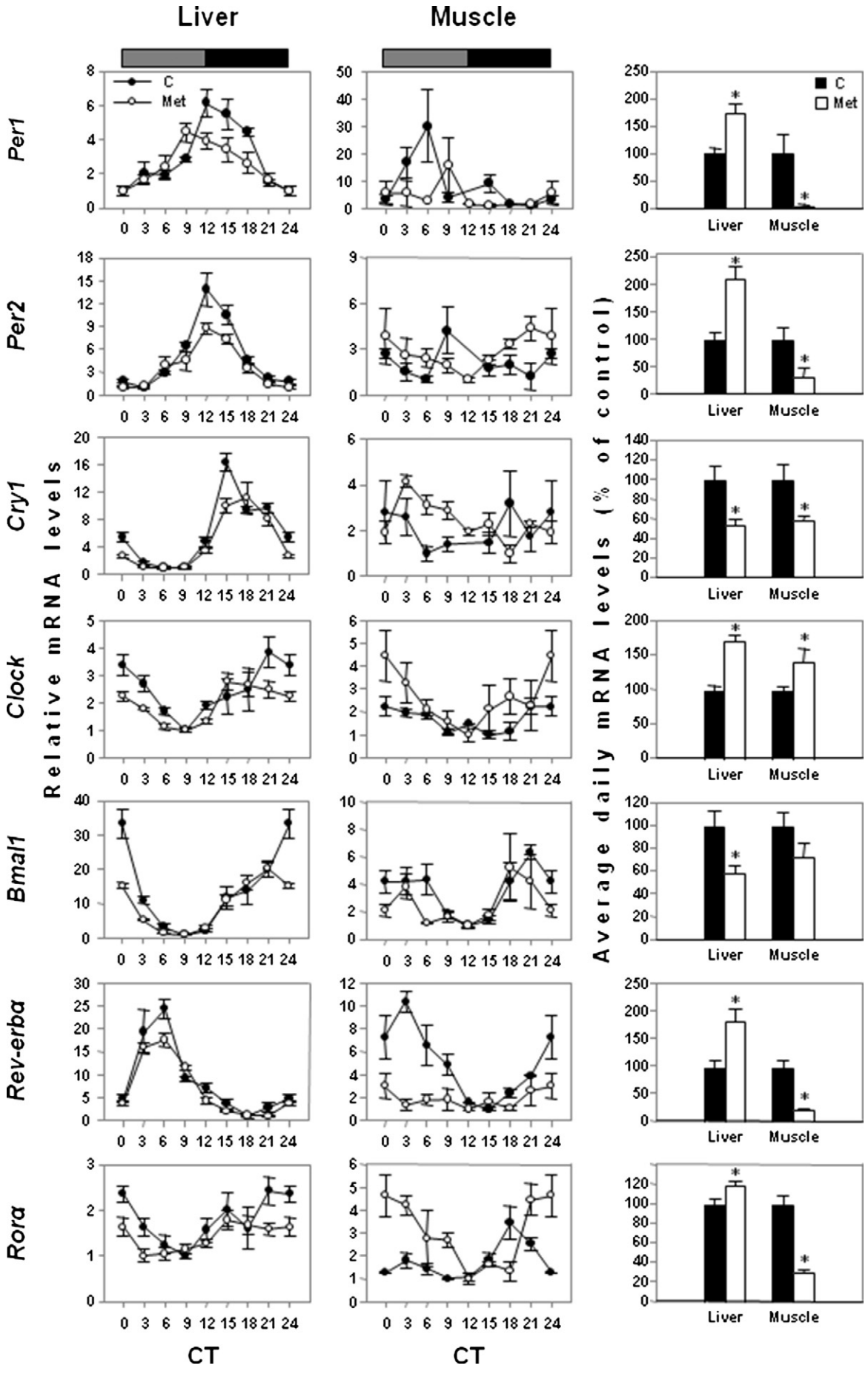
3.1. Body weight, food intake and daily locomotor activity of metformin-treated mice

Mean body weight (Fig. S1A) and food intake (Fig. S1B) throughout the experiment and on the last day (Table 1) did not differ between the metformin-treated and control mice. There was a small but significant reduction in water intake in the metformin-treated group (Student's *t*-test, $p<0.05$) (Table 1). To study the effect on the overall daily rhythms, we evaluated the effect of metformin treatment on food intake rhythms and locomotor activity. Metformin administration did not affect mouse 24-h food intake rhythms (Fig. S1C) or locomotor activity (Fig. S1D) under light–dark (LD) conditions. Under total dark (DD) conditions both groups exhibited free-running (Fig. S1D) with no effect on the endogenous circadian period (*tau*) ($tau_{control} = 23.8 \pm 0.03$ h, $tau_{metformin} = 23.7 \pm 0.09$ h).

3.2. Serum measurements of metformin-treated mice

As expected from healthy lean mice, fasting glucose was similar throughout the experiment (Fig. S1E) and on the last day (Table 1). Similarly, total cholesterol and high density lipoprotein (HDL) levels did not differ between the groups (Table 1). Also triglyceride (TG) levels did not differ between the groups (Table 1), and they exhibited a robust daily rhythm with a peak at CT5 (Cosinor analysis, $p<0.05$), toward the middle of the subjective day, in both groups (Fig. 1A). Insulin levels did not differ between the groups, but glucagon levels were reduced (Student's *t*-test, $p<0.001$) (Table 1). As glucose and insulin levels did not differ between the groups HOMA-IR was not different (Table 1). Insulin exhibited a daily rhythm and peaked at CT8 (Cosinor analysis, $p<0.05$); metformin administration resulted in a 3-h phase advance of insulin (Fig. 1B). Glucagon did not exhibit a daily rhythm in both groups (One-way ANOVA, $p>0.05$), and its levels were consistently lower at all time-points following metformin treatment (Fig. 1C). Ghrelin levels did not differ between the groups, but leptin levels were increased (Student's *t*-test, $p<0.01$) in metformin-treated mice (Table 1). Metformin treatment also resulted in a 6 h phase advance of leptin expression, from the beginning of the subjective night to the end of the subjective day (Cosinor analysis, $p<0.05$) (Fig. 1D). The amplitude of leptin was increased by 50% (Cosinor analysis, $p<0.05$) (Fig. 1D), corresponding to the overall increased daily levels (Table 1). The phase

Fig. 3. Effect of metformin on clock gene expression in the liver and muscle. Expression levels of *Per1*, *Per2*, *Cry1*, *Clock*, *Bmal1*, *Rev-erbα* and *RORα*. Liver and gastrocnemius muscle were collected in total darkness every 3 h around the circadian cycle from control (C, black circles and columns) or metformin-treated (Met, white circles and columns) mice. Total RNA was reverse-transcribed and its levels were determined by real-time PCR. For total daily levels, all time-points were averaged. The white and gray bars designate the subjective day and night, respectively. Values are mean ± SE, n = 3 per time-point in each group. Asterisk denotes significant difference ($p<0.05$). CT, circadian time.


Liver
Muscle
Per1
Per2
Cry1
Clock
Bmal1
Rev-erbα
Rora
C
Met
Relative mRNA levels
Average daily mRNA levels (% of control)
CT
CT
Liver
Muscle

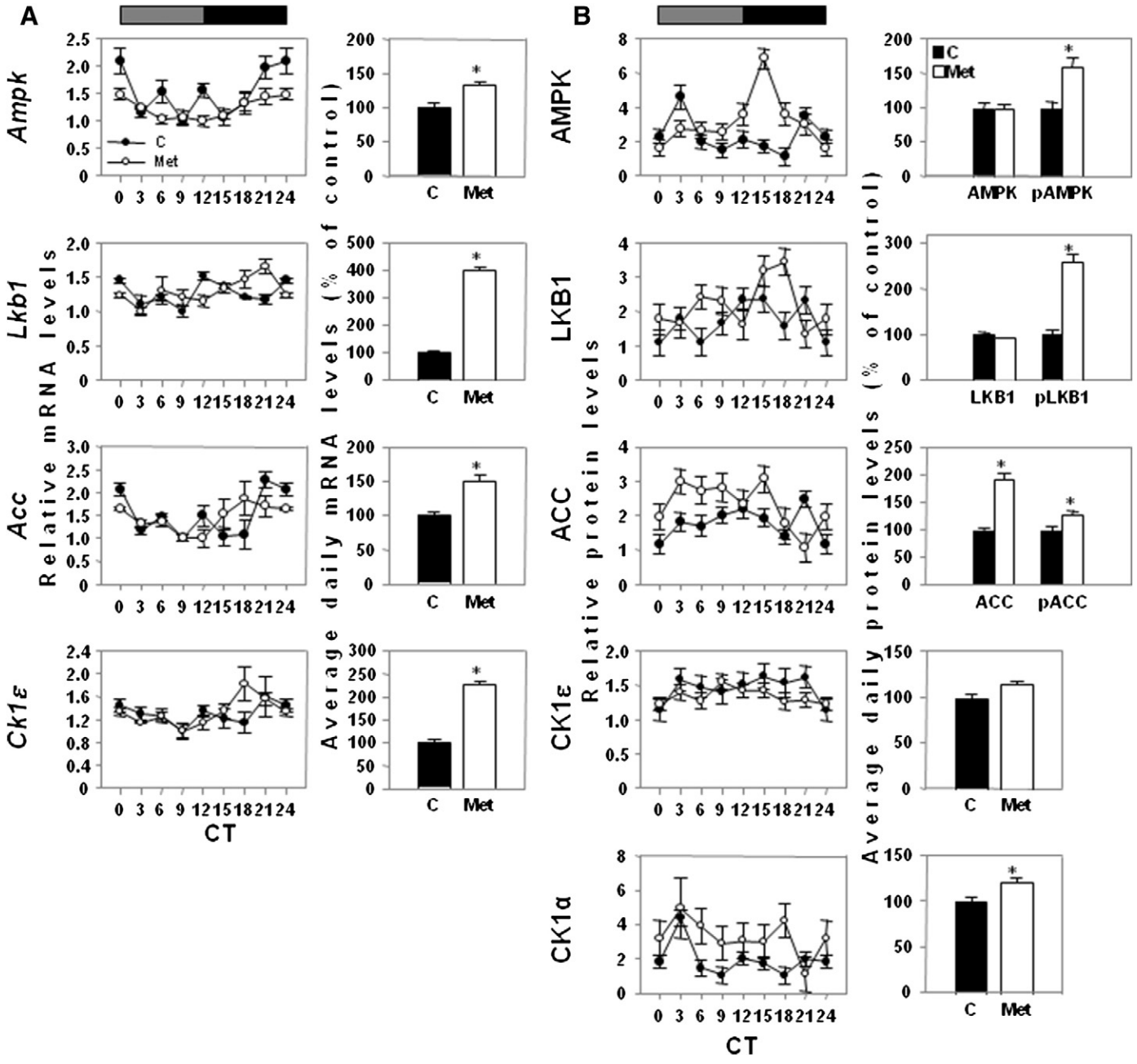


Fig. 4. Effect of metformin on circadian rhythms and average mRNA and protein levels of the AMPK pathway in the liver. A. *Ampk*, *Lkb1*, *Acc* and *Ck1ε* mRNA oscillation and daily average levels. B. AMPK, LKB1, ACC, CK1ε and CK1α protein oscillation and daily average levels. Liver was collected in total darkness every 3 h around the circadian cycle from control (C, black circles and columns) or metformin-treated (Met, white circles and columns) mice. Total RNA was extracted, reverse-transcribed and expression levels were determined by real-time PCR. Protein levels were analyzed by Western blotting and quantified using actin as loading control. The white and gray bars designate the subjective day and night, respectively. Values are mean ± SE, n = 3 per time-point in each group. Asterisk denotes significant difference ($p<0.05$). CT, circadian time.

of ghrelin was not affected by metformin treatment with a peak at CT17 during the subjective night in both groups, yet its amplitude decreased by 26% (One-way ANOVA, $p<0.05$) (Fig. 1E).

3.3. Effect of metformin on hypothalamic AMPK

We next tested the effect of metformin on hypothalamic AMPK, as AMPK is an energy gauge in this brain region. Metformin treatment resulted in reduced AMPK levels and a reduction in its active phosphorylated form (pAMPK) (Student's *t*-test, $p<0.01$) (Fig. 2A). Metformin also resulted in a 3-h phase advance in AMPK circadian oscillation (Cosinor analysis, $p<0.05$) (Fig. 2B), although pAMPK rhythmicity was unaffected (Fig. 2C).

3.4. Effect of metformin on clock genes in the liver and muscle

All clock genes oscillated in the liver and muscle in the presence or absence of metformin (Fig. 3, Table S2). Analysis of clock gene expression revealed that liver *Per1*, *Clock*, *Bmal1* and *Rorα* and muscle *Bmal1* and *Rev-erbα* exhibited a 3-h phase advance after metformin treatment (Cosinor analysis, $p<0.05$) (Fig. 3, Tables S2 and S3). Metformin did not alter the phase of liver *Cry1* and *Rev-erbα* and muscle *Clock* (Cosinor analysis, $p<0.05$). Liver *Per2* and muscle *Per1*, *Rora* and *Cry1* displayed a phase delay as a result of metformin treatment and muscle *Per2* showed a 12-h phase shift (Cosinor analysis, $p<0.05$) (Fig. 3, Tables S2 and S3).

We then looked into the daily levels of clock gene expression (Fig. 3, Table S3). Liver *Per1*, *Per2* and *Clock* mRNA daily levels increased

(Student's *t*-test, $p<0.01$), indicating increased activity of the CLOCK: BMAL1-mediated expression. Liver *Bmal1* and *Cry1* mRNA daily levels decreased as a result of metformin treatment (Student's *t*-test, $p<0.01$). These results were corroborated by a higher increase in the levels of *Rev-erbα* (Student's *t*-test, $p<0.01$), the negative regulator of *Bmal1* expression, than those of *Rora*, the positive regulator of *Bmal1* expression (Fig. 3, Table S3). In the muscle, the daily levels of most genes were reduced (Student's *t*-test, $p<0.05$), while *Clock* mRNA levels were elevated (Student's *t*-test, $p<0.05$) and *Bmal1* did not differ between the groups (Fig. 3, Table S3).

3.5. Effect of metformin on AMPK, LKB1, ACC and CKI in the liver and muscle

To study the effect of metformin on metabolism, we first measured AMPK mRNA and protein levels. Liver *Ampk* mRNA peaked at the end of the subjective night (Cosinor analysis, $p<0.05$) and metformin did not affect the expression phase (Fig. 4A, Tables S2 and S3). AMPK protein levels in control mice oscillated with a peak at CT2, whereas in the metformin-treated group AMPK exhibited robust oscillation with a peak at CT14 accompanied by a 40% increase in the amplitude (One-way ANOVA, $p<0.05$) (Fig. 4B, Table S3). Liver *Ampk* mRNA daily levels

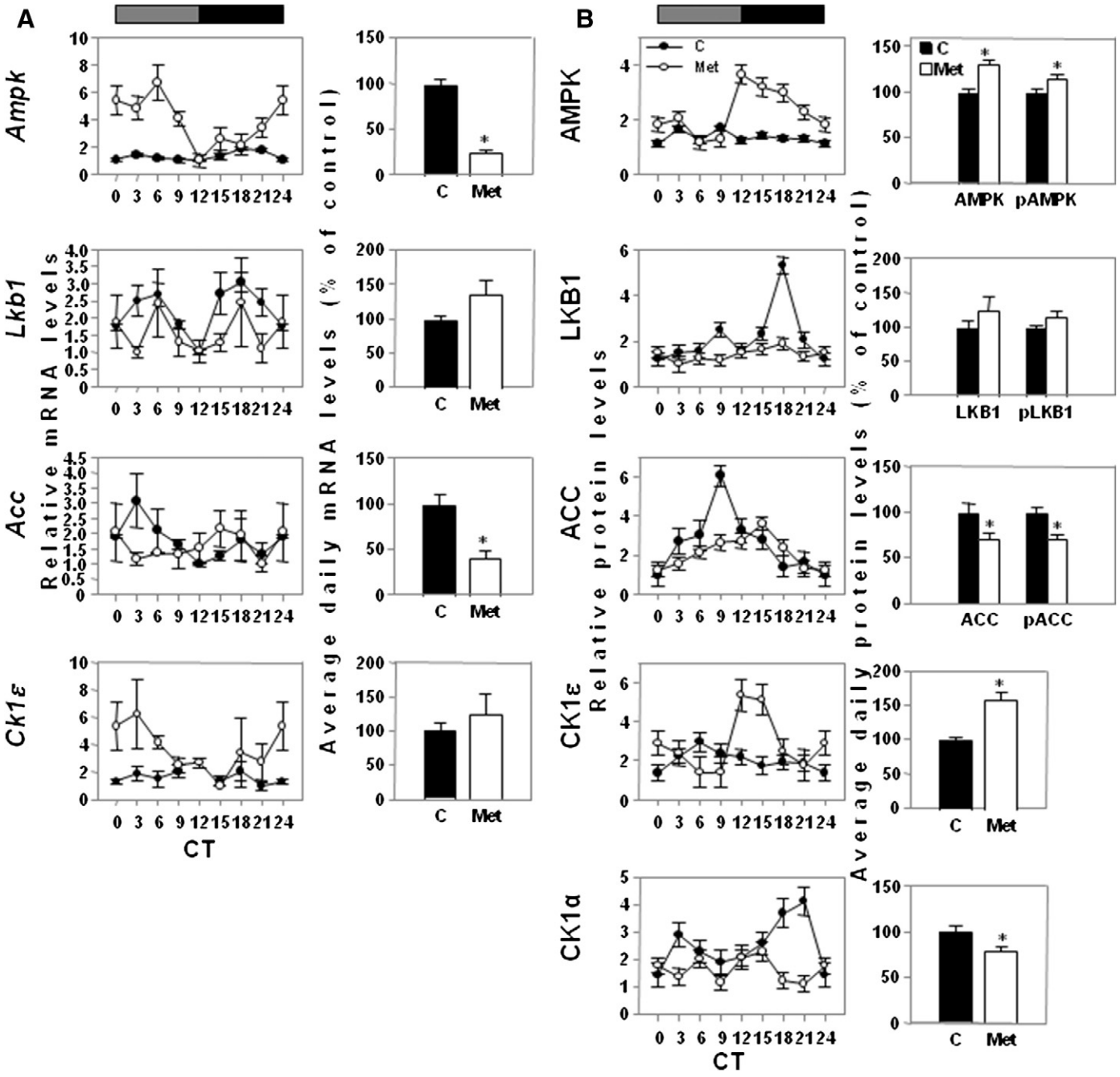


Fig. 5. Effect of metformin on circadian rhythms and average mRNA and protein levels of the AMPK pathway in the muscle. A. *Ampk*, *Lkb1*, *Acc* and *Ck1ε* mRNA oscillation and daily average levels. B. AMPK, LKB1, ACC, CK1ε and CK1α protein oscillation and daily average levels. Gastrocnemius muscle was collected in total darkness every 3 h around the circadian cycle from control (C, black circles and columns) or metformin-treated (Met, white circles and columns) mice. Total RNA was extracted, reverse-transcribed and expression levels were determined by real-time PCR. Protein levels were analyzed by Western blotting and quantified using actin as loading control. The white and gray bars designate the subjective day and night, respectively. Values are mean ± SE, n = 3 per time-point in each group. Asterisk denotes significant difference ($p<0.05$). CT, circadian time.

were significantly increased as well as pAMPK levels (Student's *t*-test, $p<0.01$) (Fig. 4, Table S3) indicating increased activation of AMPK. In the muscle, metformin treatment resulted in decreased *Ampk* mRNA expression levels (Student's *t*-test, $p<0.05$), but increased amplitude (Cosinor analysis, $p<0.05$) (Fig. 5A, Table S3). AMPK protein also showed increased amplitude (Cosinor analysis, $p<0.05$) alongside elevated daily levels of AMPK and pAMPK protein (Fig. 5B, Table S3). In addition, metformin treatment resulted in a phase delay of 6 h in mRNA and 9 h in protein expression (Fig. 5, Tables S2 and S3).

We then measured the mRNA and protein levels of the AMPK kinase, LKB1. In the absence of metformin, liver *Lkb1* mRNA displayed a bi-phasic expression with peaks at CT1 and CT13, while protein levels did

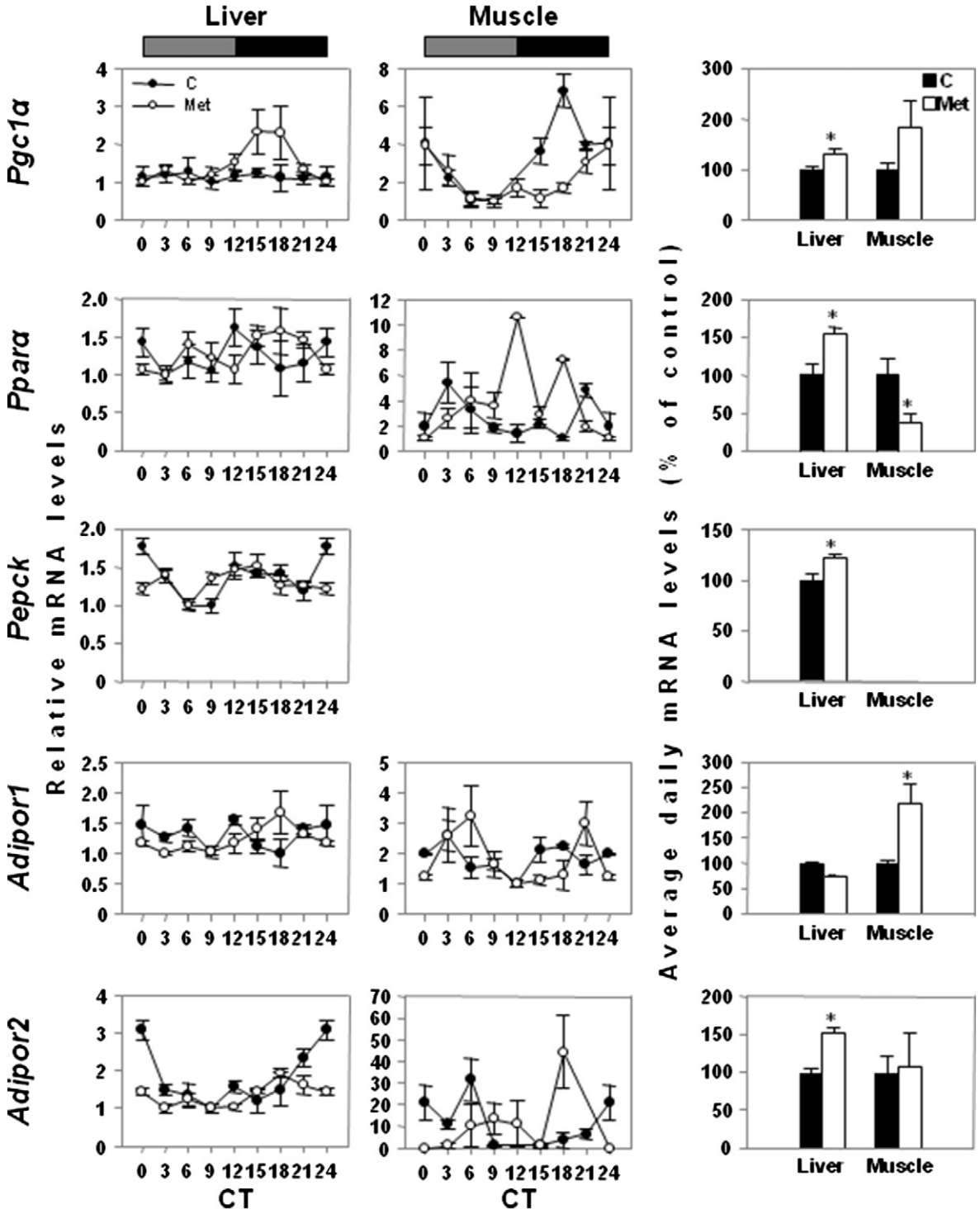


Fig. 6. Effect of metformin on metabolic genes expression in the liver and muscle. *Pgc1α*, *mPparα*, *mPepck*, *mAdipor1* and *mAdipor2* mRNA oscillation and daily average levels were measured in the liver and gastrocnemius muscle collected in total darkness every 3 h around the circadian cycle from control (C, black circles and columns) or metformin-treated (Met, white circles and columns) mice. Total RNA was reverse transcribed and its levels were determined by real-time PCR. For total daily levels, all time-points were averaged. The white and gray bars designate the subjective day and night, respectively. Values are mean ± SE, n = 3 per time-point in each group. Asterisk denotes significant difference ($p<0.05$). CT, circadian time.

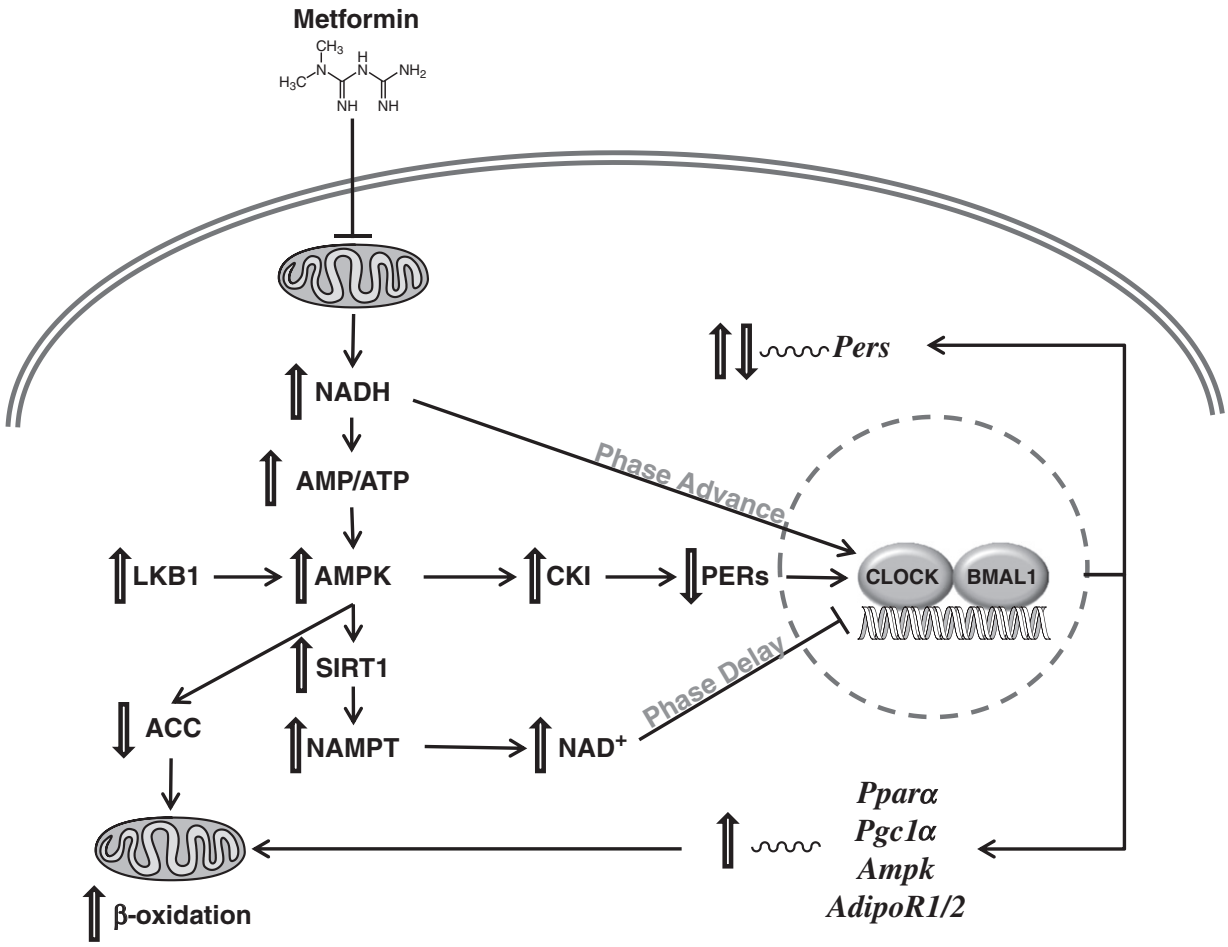


Fig. 7. Effect of metformin on metabolism and the circadian clock. Metformin blocks mitochondria Complex I leading to increased NADH and AMP levels. This increase results in CLOCK:BMAL1 promoter binding, which leads to phase advance. High AMP levels activate AMPK, which phosphorylates CKI leading to PER protein degradation and relief of CLOCK:BMAL1 inhibition. This relief causes increased CLOCK:BMAL1-mediated transcription, which, in turn, leads to a phase advance. AMPK also phosphorylates and, thus, inhibits ACC leading to increased fatty acid oxidation and decreased fatty acid synthesis. AMPK activates SIRT1 and NAMPT, which leads to increased levels of NAD^+. High levels of NAD^+ reduce CLOCK:BMAL1 promoter binding, which leads to a phase delay. The tissue-specific balance between NAMPT and AMPK activities determines the phase shift in the clock.

not show a significant bi-phasic expression (One-way ANOVA, $p > 0.05$) (Fig. 4, Table S2). Metformin resulted in a 5-h phase advance of liver *Lkb1* mRNA and induced a significant bi-phasic expression of LKB1 protein (Cosinor analysis, $p < 0.01$) (Fig. 4, Table S2). The daily mRNA levels were increased 4-fold after metformin treatment (Student's *t*-test, $p < 0.01$) (Fig. 4A, Table S3). Total LKB1 protein daily levels did not differ between the groups, but pLKB1 levels were significantly increased in the metformin group (Student's *t*-test, $p < 0.01$) (Fig. 4B), indicating increased activation of LKB1. In the muscle, *Lkb1* mRNA and LKB1 protein exhibited a bi-phasic rhythm (Cosinor analysis, $p < 0.01$). Metformin treatment resulted in the loss of daily rhythmicity of both mRNA and protein (Fig. 5). Total LKB1 remained unchanged after metformin treatment (Student's *t*-test, $p > 0.05$), although pLKB1 levels showed a non-significant 18% increase (Student's *t*-test, $p = 0.067$) (Fig. 5B).

We next measured the mRNA and protein levels of the AMPK substrate, ACC. *Acc* mRNA exhibited a daily oscillation with a peak at CT23, while metformin treatment resulted in a 3-h phase advance (Cosinor analysis, $p < 0.05$) (Fig. 4A, Tables S2 and S3). ACC protein did not oscillate in the control group, while metformin resulted in a significant daily rhythm with a peak at CT8. *Acc* mRNA, ACC and pACC protein daily levels were significantly increased in the metformin group (Student's *t*-test, $p < 0.01$) (Fig. 4). In the muscle, *Acc* mRNA levels of the control group oscillated with a peak at CT5 (Cosinor analysis, $p < 0.05$), while in the metformin group *Acc* did not exhibit circadian rhythm (One-way ANOVA, $p > 0.05$) (Fig. 5A). Like in the liver, ACC protein did not oscillate in the muscle of control mice, but oscillation was induced by metformin treatment with a peak at CT14. The daily levels of *Acc* mRNA, ACC and pACC protein decreased following metformin administration (Student's *t*-test, $p < 0.05$), suggesting inhibited fatty acid synthesis (Fig. 5, Table S3).

We also measured the mRNA and protein levels of another AMPK substrate, CKIε, the kinase that phosphorylates the PER proteins and directs them for degradation. Liver *Ckiε* mRNA exhibited a significant oscillation only in the metformin group (One-way ANOVA, $p < 0.01$). mRNA levels were significantly increased in the metformin group (Student's *t*-test, $p < 0.01$) (Fig. 4A). The protein levels did not oscillate in both groups (One-way ANOVA, $p > 0.05$) and the daily levels remained unchanged (Fig. 4B). CKIα protein exhibited a daily rhythm with a peak at CT2 (One-way ANOVA, $p < 0.05$). Metformin treatment resulted in a loss of CKIα oscillation (One-way ANOVA, $p < 0.05$), while its levels increased (Student's *t*-test, $p < 0.01$) (Fig. 4B, Table S3). In the muscle, similarly to the liver, *Ckiε* mRNA levels exhibited a circadian rhythm only following metformin administration with a peak at CT2 (Cosinor analysis, $p < 0.02$) (Fig. 5A, Table S2). A similar effect of induced oscillation following metformin treatment was observed in CKIε protein levels. While *Ckiε* mRNA levels did not differ between the groups, CKIε protein levels increased (Student's *t*-test, $p < 0.05$) in the metformin group (Fig. 5). CKIα protein oscillation was accompanied by a reduction

in total protein levels in the metformin group (Student's *t*-test, $p<0.05$) (Fig. 5).

3.6. Effect of metformin on PGC1α, PPARα, adiponectin receptors, and PEPCK expression

In the liver, metformin administration resulted in the oscillation of *Pgc1a* mRNA (One-way ANOVA, $p<0.01$), a key co-activator of PPARα, while *Pparα* mRNA did not oscillate (One-way ANOVA, $p>0.05$) (Fig. 6). In the muscle, metformin treatment resulted in a 3-h phase delay and decreased amplitude of *Pgc1a* (Cosinor analysis, $p<0.05$), and induced a robust circadian rhythm of *Ppara* (One-way ANOVA, $p<0.01$) (Fig. 6, Table S3). Both *Pgc1a* and *Pparα* daily mean levels were elevated in the liver (Student's *t*-test, $p<0.01$), while in the muscle *Ppara* was reduced (Student's *t*-test, $p<0.01$) and *Pgc1a* levels were not affected by metformin. The phase of *Pepck*, a key gluconeogenic enzyme in the liver, exhibited a 3-h phase advance (Cosinor analysis, $p<0.05$) and its daily levels were slightly, but significantly, elevated in the metformin-treated group (Student's *t*-test, $p<0.05$) (Fig. 6, Table S3).

We next measured signaling of adiponectin, an adipokine that leads to AMPK activation in the muscle and liver. The adiponectin receptor *Adipor1* mRNA did not exhibit daily rhythmicity in the liver (One-way ANOVA, $p>0.05$), while *AdipoR2* mRNA exhibited marked oscillation (One-way ANOVA, $p<0.01$) and metformin administration resulted in a 3-h phase advance (Fig. 6, Table S3). There was also a 55% increase in the mean daily levels of *Adipor2* mRNA in the liver (Student's *t*-test, $p<0.01$) (Fig. 6, Table S3). In the muscle, both receptors did not exhibit a daily rhythm (One-way ANOVA, $p>0.05$). The levels of *Adipor1* mRNA were significantly elevated in metformin-treated mice (Student's *t*-test, $p<0.05$) while *Adipor2* mRNA levels remained unchanged (Fig. 6, Table S3).

4. Discussion

In the current study, we investigated the effect of metformin treatment on both metabolic and circadian rhythms. Although we performed the experiment on young, lean healthy mice to avoid the disruptive effect of obesity or diabetes on the circadian clock, our results show profound tissue-specific effects on metabolic rhythms in the liver and muscle.

4.1. Effect of metformin on overall metabolism

As the experiment was performed on lean and healthy mice, daily food intake and body weight were not affected by metformin administration. There was a slight reduction in water consumption, probably due to the effect of metformin on the water taste. Mean daily levels of insulin, glucose and lipids in the serum were not affected after 6 weeks of metformin administration, as is expected in lean healthy mice. In contrast, the daily levels of glucagon, referred to as a hypoglycemic signal [24], were reduced. As glucagon secretion is inhibited by glucagon-like peptide 1 (GLP-1) [25], it is possible that metformin affected the small intestine to secret more GLP-1 leading to reduced glucagon secretion, as has recently been shown [26]. As it is well established that high glucagon levels act in the short-term to reduce meal size [27], we should have expected increased food intake and weight gain. However, in parallel, leptin levels were elevated (Table 1). As leptin is a long-term anorexigenic signal [28], the overall effect achieved in our experiment is that of satiety. This is further reinforced by the reduced levels of AMPK and pAMPK in the hypothalamus (Fig. 2), as reduced AMPK expression or activation in this brain area has been shown to signal satiety [14]. It is noteworthy that as the whole hypothalamus was analyzed it is possible that the various regions, such as the lateral hypothalamus or ventromedial hypothalamus, had opposing effects on AMPK levels. Thus, in lean healthy mice, the net effect of metformin is maintenance of food intake and body weight. Longer metformin treatment or treatment of obese and/or diabetic mice may have caused reduced food intake and, as a result, reduced body weight, as has been reported [29,30].

At the cellular level, metformin led to AMPK activation, in the liver via LKB1 and in the muscle by another kinase, presumably CaMKK [31]. Activation of ACC, the AMPK substrate, decreased in the liver as expected by the rise in pAMPK levels (Fig. 4). In the muscle, the total levels of *Acc* mRNA, ACC protein and its amplitude decreased, implying reduced ACC activity. ACC reduced expression or inhibition leads to reduced fatty acid synthesis and increased fatty acid oxidation (Fig. 7). In addition, increased sensitivity to fatty acid and insulin was achieved, as reflected by increased liver *Pparα* and *Pgc1α* mRNA levels and the robust increase in muscle *Pparα* mRNA amplitude. Although AMPK activation has been shown to inhibit gluconeogenesis [32], liver *Pepck* levels were slightly elevated compared to the control group. These results can be explained by the elevated *Pparα* and *Pgc1α* mRNA levels, as both PPARα and PGC1α have been implicated in the regulation of *Pepck* expression [33,34]. The elevated gluconeogenic activity would prevent hypoglycemia after fasting, a well described attribute of metformin [35]. Metformin also affected the expression of adiponectin receptors. AdipoR1, the abundant isoforms in the muscle, and AdipoR2, the predominant isoform in the liver, were each upregulated in their corresponding tissue. These results suggest that metformin leads to increased tissue-specific adiponectin receptor expression, which would signal activation of AMPK, fatty acid oxidation and inhibited fatty acid synthesis once adiponectin binds to its receptors [36]. Overall, the metabolic profile shows increased fatty acid oxidation, inhibited fatty acid synthesis and insulin sensitivity both in the liver and the muscle.

4.2. Effect of metformin on circadian rhythms

Insulin, leptin, glucagon, ghrelin and triglycerides exhibited a clear endogenous rhythm both in the control and metformin-treated mice (Fig. 1). Leptin and ghrelin exhibited reciprocal rhythms with leptin high in the inactive period and ghrelin high in the active period, as has been previously described [37–39]. Insulin and leptin exhibited a phase advance in the metformin-treated mice indicative of metformin effect on the circadian mechanism. Metformin also induced oscillation of liver *Pgc1α* and *CkIε* and muscle *Pparα*, rendering a more robust circadian profile. This further demonstrates the effect of metformin on the circadian mechanism. Synchronization of metabolic functions leads eventually to a healthier phenotype [40]. Indeed, activated LKB1 and CK1 were previously indicated to be involved in tumor suppression [41] and cancer development [42], while metformin has recently been investigated as a putative enhancer of chemotherapy [43].

Recently it has been shown that metformin crosses the blood–brain barrier and accumulates in the brain of rodents [44]. Our results indicate that metformin does not affect the period of the central circadian clock located in the SCN, as reflected by no change in locomotor activity, a direct readout of the central circadian clock. However, assessment of the circadian expression of the core clock mechanism and metabolic genes in the peripheral tissues revealed that metformin has tissue-specific effects.

In the liver, most clock and metabolic genes showed a phase advance as a result of metformin treatment. The elevated AMPK activity by metformin results in a shortened half-life of the negative feedback loop and increased activity of the positive-loop of the circadian clock [6]. Most recently, it has been shown that AMPK enhances the activity of SIRT1, an NAD^+-dependent histone deacetylase, by increasing cellular NAD^+ levels, resulting in the deacetylation and modulation of the activity of downstream SIRT1 targets, such as PPARγ and PGC-1α, key factors that regulate the core molecular clock [45]. Activated SIRT1 also deacetylates BMAL1, PER2, and histones [46] leading to relief of PER:CRY-mediated inhibition and promoting a phase advance. These results are in agreement with our previous report that fasting, which also leads to AMPK and SIRT1 activation, resulted in phase advances [20]. Although the levels of liver CKIε, the kinase that phosphorylates the PER proteins

leading to their degradation, remained unchanged, CKIα protein levels were elevated. Indeed, it has been recently reported that other members of the CKI family are involved in regulation of the clock mechanism [47]. The phase advance mediated by metformin can also be explained by the finding that metformin treatment results in reduced cellular redox potential, possibly through inhibition of Complex I in the mitochondria [48]. Inhibition of the respiratory chain would lead to increased levels of reduced nicotinamide adenine dinucleotide (NADH). As CLOCK and BMAL1 bind efficiently to E-box sequences in the presence of high NADH [49], increased NADH levels, as a result of metformin treatment, would lead to enhanced activity of CLOCK:BMAL1-mediated expression (Fig. 7). Indeed, metformin resulted in increased daily levels of *Per1* and *Per2* mRNA in the liver. In parallel, *Clock* expression also increased. There was a decrease in *Bmal1* and *Cry1* expression, presumably as a result of the marked increase in *Rev-erbα*, a known repressor of both genes [4,50].

In the muscle, while some genes presented a phase advance, similarly to the liver, others exhibited a phase delay. This implies a circadian regulatory mechanism slightly different than that of the liver. The increased muscle pAMPK and CKIε protein levels, the kinase responsible for PER proteins phosphorylation and degradation, support AMPK involvement in the circadian clock mechanism, similarly to what has been found in the liver. However, it was recently demonstrated that AMPK activation in the muscle, e.g., by metformin, leads to the activation of nicotinamide phosphoryl-transferase (NAMPT), the rate limiting enzyme in NAD^+ synthesis [45,51]. In addition, SIRT1, which is activated by AMPK, is recruited to the *Nampt* promoter and contributes to the circadian synthesis of its own coenzyme [52]. In turn, high NAD^+ levels inhibit DNA binding of CLOCK:BMAL1, which results in decreased expression and reduced daily levels of *Per1*, *Per2* and other clock-controlled genes, as we have found in this study. Thus, in the muscle, we have a dual effect of metformin, inhibition of Complex I, which leads to increased NADH levels, and activation of NAMPT, which leads to increased NAD^+ levels. However, unlike metformin, which increases both NAD^+ and NADH levels, activation of SIRT1 alone leads to an increase only in NAD^+ levels. This dual effect after metformin treatment results in a more complex effect on CLOCK:BMAL1-mediated expression leading to an overall higher ratio of NAD^+/NADH, resulting, in turn, mainly in phase delays.

Although NAMPT activation may also be a factor in the liver, the balance between both effects achieved with metformin treatment possibly determines the phase of circadian rhythms in a tissue-specific manner (Fig. 7). Although metformin affected circadian rhythms differently in the liver vs. in the muscle, it had the same overall effect on metabolism in both tissues. As genes are expressed at different levels in the liver vs. in the muscle, e.g., NAMPT, AMPK or SIRT1, it seems that the differential effect on circadian rhythmicity as a result of metformin treatment is unavoidable. However, as each peripheral tissue has a slightly different endogenous phase of clock gene expression [53], the effect seen with metformin should not reflect a pathophysiological situation. This is corroborated by the same overall metabolic effect shown in both tissues. In addition, it was recently shown that AMPK activity was decreased in white adipose tissue (WAT) of high-fat-fed mice, in association with suppressed expression of the core circadian component the CLOCK protein [54]. However, metformin opposed this effect and led to both AMPK activation and increased CLOCK expression, as was found in this study.

5. Conclusions

Metformin induces the activation of AMPK either by LKB1 in the liver and/or by other kinases, such as CaMKK, in the muscle. In turn, AMPK phosphorylates both ACC and CKI enzymes leading to fatty acid oxidation and enhanced activity of the positive loop of the circadian clock, respectively. This drives the expression of both clock and metabolic genes. The different NAD^+/NADH ratios may explain the differences in circadian expression phase in the liver vs. in the muscle.

Author contribution

Conceived and designed the research: MB, OF, ZM. Performed the experiments: MB, LH, RG, NC. Analyzed the data: MB, LH, RG. Wrote the paper: MB, OF, ZM.

Conflict of interest

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2012.08.005.

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